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MICROCHEMICAL ANALYSIS OF OTOLITHS TO TO DETERMINE LIFE HIST. PATTERNS IN INTERIOR SALMONIDS USING SECONDARY ION MASS SPECTROMETRY

Microchemical Analysis of Otoliths to Determine Life History Patterns in Interior Salmonids Using Secondary Ion Mass Spectrometry

Phase Two

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Introduction:

Analysis of the chemical composition of fish otoliths, otolith microchemistry, is a relatively new field in fisheries science and has already been applied to many different areas in this field of research. Stock identification has been one of the main applications of otolith microchemistry. Another application has been to determine the environmental and early life history of fish. For example, Rieman et al. (1994) used the differences in the ratio of strontium to calcium (Sr:Ca) to discriminate between anadromous and resident sockeye salmon *Oncorhynchus nerka*. To date, much of the work in otolith microchemistry has been done using marine and anadromous species of fish. Studies on the application of otolith microchemistry techniques to freshwater systems have been limited because freshwater generally has lower concentrations of elements than saltwater. Also, until recently instruments sensitive enough to detect very low concentrations of elements have not been available.

The overall goal of this study was to evaluate the efficacy of Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) in the microchemical analysis of the otoliths of inland salmonids. To fully evaluate the utility of both this instrument and of the use of microchemistry to differentiate stock and life history differences in freshwater salmonids, our specific objectives were to: 1) test the precision and accuracy of ToF-SIMS; and 2) assess its applicability to detecting microchemical differences in fishes from freshwater systems. We tested the ability of the instrument to detect microchemical differences of otoliths of rainbow trout *O. mykiss* and westslope cutthroat trout *O. clarki lewisi* in two geographically separate and geologically different drainages, the Missouri River, Montana, and the Coeur d'Alene River, Idaho. ToF-SIMS has been identified as an instrument that is more sensitive than electron microprobes for the determination of the chemical composition of otoliths (Campana et al. 1997). Other advantages of ToF-SIMS include its ability to collect information on multiple elements and their isotopes simultaneously, a feature not attainable with electron or laser microprobes, and to non-destructively analyze whole otolith samples with only a small amount of preparation. Because of the non-destructive properties of beam-based techniques, they are extremely useful in gathering temporal changes in otolith composition. One of the disadvantages of ToF-SIMS is that it is a surface analysis technique and thus uses a low energy ion beam to sputter the sample surface. Therefore only a small amount of sample is produced that can be analyzed by the mass detector. This makes it difficult to quantify the elements that are detected. Thus another objective of our study was to compare SIMS results with more quantitative methods. In addition, this type of instrument has not be previously used for otolith microchemistry, therefore much time has been needed to develop the sample preparation techniques and learn how to operate the instrument in order to get consistent spectra.

Methods (General):

Otolith preparation: Otoliths were extracted from the fish shortly after capture or were frozen as soon as possible until a time when the otoliths could be extracted. Upon extraction, the otoliths were cleaned with Milli-Q water (deionized, reverse osmosis, and millipore filtered), and stored dry in acid washed polyethylene vials. In preparation for microchemical analysis with ToF-SIMS, otoliths were mounted on glass slides, ground with 2,000 grit wet/dry sandpaper, and polished with 0.3-μm AlO_3 polishing powder to produce a thin sagittal section of the otolith. These preparation methods were similar to the standard otolith preparation techniques described by Secor et al. (1992). Before microchemical analysis, the otoliths were cleaned in a bath of analytical grade hexane followed by a bath of analytical grade methanol. Once clean, the otoliths were coated with 15 nm of Au/Pd. A final cleaning in a hexane bath followed by a methanol bath was done before the samples were analyzed in order to remove impurities.

ToF-SIMS analysis: Analyses were conducted in a Phi-Evan's triple focusing time-of-flight secondary ion mass spectroscopy system at the Image and Chemical Analysis Laboratory of the Montana State University Physics Department (ICAL). Prior to collecting a spectrum, the Au/Pd coating and surface contaminants were removed by sputtering a 60 x 60-μm area in the region of interest for 4 minutes. Spectra were collected using a pulsed energetic (15 keV) primary Ga^+ ion beam, focused to 9 x 9-μm, that was aimed in the center of the area where the Au/Pd coating had been removed. Acquisition time for each analysis was 5 minutes. Total ion counts were measured for ^{40}Ca, its two most abundant isotopes ^{42}Ca and ^{44}Ca, and Sr. During the development of methods for the ToF-SIMS we suspected that the mass analyzer was being swamped with too many ^{40}Ca ions and was therefore not able to detect and record every ^{40}Ca ion. A comparison of ^{44}Ca ion counts to ^{40}Ca counts showed that we did not have a linear relation with as slope of 0.0215 as was expected. This value was based on the relative abundance of these two naturally occurring isotopes (2.09% and 96.94% respectively). Comparisons of ^{42}Ca (another naturally occurring Ca isotope) and ^{44}Ca indicated that their abundances were being detected by ToF-SIMS in the same ratio as they naturally occur. From this, we concluded that ^{40}Ca was indeed swamping the ToF-SIMS mass analyzer and not all of the ions were being detected, thus artificially elevating the Sr:Ca ratios. To correct for this, we used the ^{44}Ca total ion count to calculate Sr:Ca ratios. In order to make the ToF-SIMS Sr:Ca ratios comparable to those obtained using other instruments, we estimated what the ^{40}Ca ion count would be if all of the ^{40}Ca ions had been detected. This was done by dividing the ^{44}Ca counts by 0.0215 (the ^{44}Ca:^{40}Ca ratio based on their relative abundances).

Results by Objective:

1. Develop standards for testing the accuracy and precision of Secondary Ion Mass Spectrometry in the determination of the chemical composition of otoliths

A. Development and testing of standard

Preliminary analyses of otoliths using ToF-SIMS in Phase One of this study raised questions regarding the consistency and of this and other microprobe instruments, from one session to the next. Data from Phase One of the study raised the question of whether the sample variability that we observed was a result of true sample variation or, alternatively, to sampling variation from one session to the next due to differences in instrument tuning. In order to answer this question, we developed "standard" samples that were used to ensure the ToF-SIMS produced consistent results. The use of standards to calibrate the instruments and to measure the performance characteristics of the various instruments used for otolith microchemical analysis has received much attention lately. Standard reference materials, such as those produced by The National Institute of Standards and Technology, are typically used to calibrate the instruments. However, these standards were not developed for otolith microchemistry and might introduce bias into the results (Campana et al. 1997). Ideally, primary standards need to be developed with matrices that match otoliths; however, this has met with limited success (e.g., Campana et al. 1997).

Our objective was to produce a standard using otoliths so that the matrix would be the same as natural otoliths. To make our standards, otoliths from about 500 hatchery-raised rainbow trout *O. mykiss* were extracted and cleaned using methods similar to Fowler et al. (1995). Glass probes were used to extract the otoliths and great care was taken to avoid any contact with metal (the otoliths, probes, or any other item used). Each otolith had excess tissue teased off with the probes. Otoliths were rinsed using Milli-Q water and placed in polyethylene vials. Otoliths in which vaterite had clearly replaced the normal aragonite form of $CaCO_3$ were discarded from the samples. About 200 of the otoliths (0.2 g) were ground into a fine powder using an AlO_3 mortar and pestle. The otolith powder was then mixed and sifted through 5-μm mesh nylon cloth. The artificial otoliths were made by pressing a small portion of the otolith powder between two pieces of acetate in an infrared spectroscopy press. The discs were mounted on glass slides using thermoplastic glue and polished with 0.3-μm AlO_3 polishing powder. The polishing removed any loose otolith material and gave a smooth sample surface, which is required for ToF-SIMS analysis. The samples were cleaned, coated and analyzed the same as other samples as described above.

A series of assays were done when the "standard" samples were first used in order to establish a baseline Sr:Ca ratio. Analyses included three areas were analyzed during a single session when the ToF-SIMS had been thoroughly tuned, and one of the sites was analyzed multiple times. The mean Sr:Ca ratio among the three sites was 0.3417×10^{-3} (SD = 0.0216×10^{-3}; 6.3% of the mean). Multiple assays of a single site resulted in a mean Sr:Ca = 0.3494×10^{-3} (SD = 0.0165×10^{-3}; 4.7% of the mean). The among site and within site mean Sr:Ca ratios were not significantly different (t-test, $n = 6$, $P = 0.50$). The following day the

same three sites on the "standard" samples were analyzed. The mean Sr:Ca ratios of these three areas were not significantly different between the two sessions (matched pair t-test, n = 3, P = 0.26). Using all of the assays from these two sessions on the ToF-SIMS, the mean Sr:Ca for "standard" otolith estimated to be 0.3311×10^{-3} (SD = 0.0216×10^{-3}).

Once these baseline values were established, the "standard" otolith sample was used at the beginning of each session on the ToF-SIMS. In most cases (9 of 15 sampling sessions), the Sr:Ca ratio of the standard fell within ± 2 SD of the mean (Figure 1). Sometimes, the initial Sr:Ca ratio of the standard fell outside of the upper and lower levels of acceptance (e.g., sessions 3, 8 and 15). After further adjustments of the instruments, the Sr:Ca ratio was generally within the ± 2 SD criteria. The variation in the results and the number of sessions that yielded unacceptable declined as we gained experience in tuning the instrument. When unacceptable readings did occur there were other signs that accompanied the unusual Sr:Ca ratio. For example, if the beam was not focused properly the peaks in the spectra would not be as sharp as when the instrument was properly tuned and occasionally, multiple peaks would form instead of the usual single peak. In cases such as this, the session was ended at that point and results omitted from further analysis.

An additional diagnostic that we used to determine that the ToF-SIMS was properly tuned was the ratio of ^{42}Ca to ^{44}Ca. As explained above, these two isotopes of Ca naturally occur and their relative abundances are 0.647 and 2.086% respectively. The ^{42}Ca:^{44}Ca ratio is therefore 0.310. We found that the mean ^{42}Ca:^{44}Ca ratio for the standard sample was 0.319 (SD = 0.0034). This data provided an additional check of each assay as the session progressed.

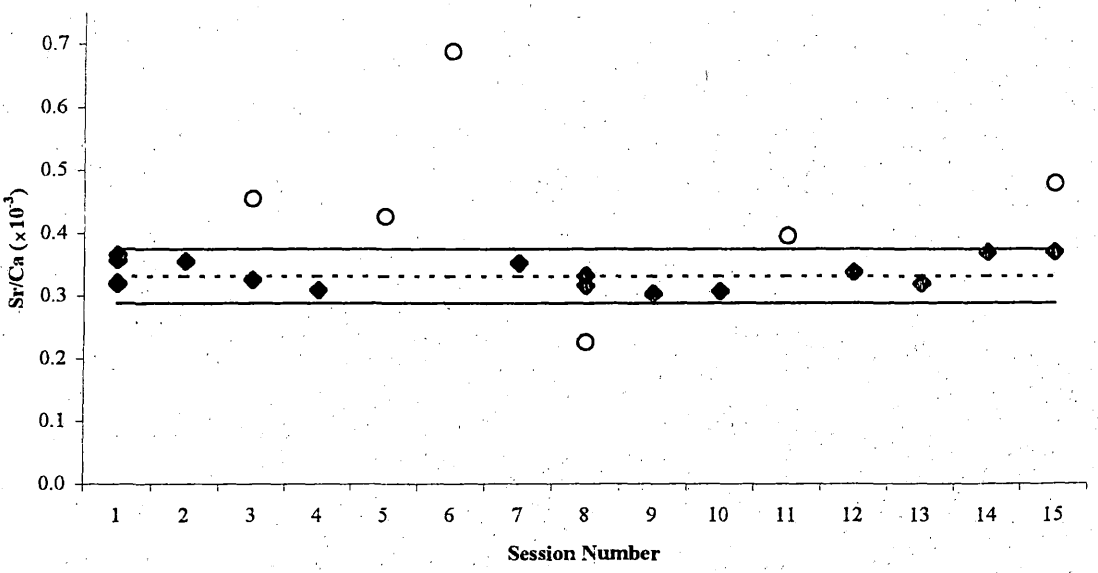

Figure 1 Sr:Ca ratios of the artificial otolith used to check for consistency in tuning of the ToF-SIMS. The dashed line is the mean Sr:Ca from the baseline analysis; the solid lines are ± 2 SD. Open circles were outside of the range of acceptability (2 SD) and those results omitted from further analysis?

B. Comparison of microprobe methods

In Phase One of this study, we analyzed a single sockeye salmon otolith that had previously been analyzed using a wavelength dispersive electron microprobe (WD-EM). The results from the ToF-SIMS analyses showed a similar pattern of higher Sr:Ca ratios in the saltwater growth region of the otolith when compared to the freshwater growth region. To further assess the accuracy of ToF-SIMS is to an accepted method of otolith microchemical analysis (WD-EM), we compared Sr:Ca ratios with the two different instruments using additional otoliths (1 sockeye and 19 Coeur d'Alene cutthroat trout). One otolith of each pair had been previously analyzed using a WD-EM, and the other otolith from the pair was used to measure Sr:Ca using ToF-SIMS. In sockeye otoliths, three to four zones were analyzed: the primordia, nucleus, freshwater growth, and saltwater growth. In cutthroat trout otoliths, two zones were sampled, the nucleus and edge. In all, 45 sites were compared between the two methods.

Comparison of the ToF-SIMS results with the WD-EM results show a significant positive linear relation between the Sr:Ca ratios obtained using the two instruments (Figure 2). While the relation yielded a r^2 of 0.79, four data points appeared to be outliers that strongly influenced the regression (dashed circle, Figure 2). These points represented the edge zones of four of the five Lavin Creek cutthroat trout. In these cases, the Sr:Ca ratios from the WD-EM analyses were about twice that obtained using the ToF-SIMS. When these samples were removed from the analysis, the r^2 increased to 0.95 and the slope of the regression increased to 0.916, indicating a near 1:1 relation between Sr:Ca ratios obtained using ToF-SIMS and WD-EM. Why the edge zone of these otoliths yielded such a higher Sr:Ca ratio with ToF-SIMS is not clear. A possibility is that there is some kind of edge effect from the thermal plastic glue used to mount the otoliths. However, in three of the otoliths at least two different edge sites were analyzed, with some being located further from the otolith/glue interface. Plus, a similar effect was not seen in other edge samples. Another possibility that we investigated was that the detector was not able to detect and record every Sr ion, as was the case with ^{40}Ca. This is probably not the case because, ion counts need to be in the millions in order for the detector to not detect all of the ions produced (R. Avci, ICAL, Montana State University, MT, personal communication). One other possibility is that the WD-EM results were inaccurate.

The precision of the ToF-SIMS was determined by sampling the same site 2 to 3 times in a subsample of the cutthroat trout otoliths. In addition, we determined the variation within an otolith zone by sampling multiple sites (2 – 3 sites) within the same zone of the otolith (i.e., nucleus, otolith edge). The WD-EM sampling of the cutthroat trout otoliths was similar to the ToF-SIMS sampling in that multiple sites were analyzed within the different otolith zones and that multiple analyses were done within a small area. Unlike ToF-SIMS, WD-EM creates a pit at the analysis spot; therefore, the beam had to be moved in order to replicate the analysis. In the case of the WD-EM samples, the nucleus was analyzed 10 times and the three locations on the edge of the otoliths were analyzed five times each, for each otolith. It was found that the mean coefficient of variation (CV) for analyses within a single area and among areas within a given zone were, on average, lower for ToF-SIMS than WD-EM (Table 1).

Table 1 Comparison of ToF-SIMS and WD-EM sample variation (CV) within a single analysis area and among analysis areas in a given otolith zone in the Coeur d'Alene River cutthroat trout. Sample sizes are in parentheses.

Coefficient of Variance (%)	Instrument	
Within a site (instrument precision)	SIMS	WD-EM
Mean	6.93 (18)	14.51 (57)
Min	0.17	1.77
Max	17.19	45.67
Among sites (zone variation)		
Mean	9.82 (15)	18.21 (38)
Min	0.93	1.22
Max	35.70	47.82

C. Comparison of SIMS instruments

In the study proposal it was noted that a group at University of California, Berkeley and Lawrence Livermore National Laboratory had begun investigating the used of SIMS to identify the tributary of origin of Chinook salmon *O. tshawytscha*. The purpose of this objective was to exchange otolith samples and verify the results obtained on the respective machines so as to provide another potential means of testing the ToF SIMS instrument. Initial contact with one of the members of the group did not seem promising so this objective was not pursued further. The U.C. Berkeley/Lawrence Livermore Lab group is using dynamic-SIMS for their otolith microchemical analysis and they expressed some concerns about using static-SIMS (i.e., ToF-SIMS). Their concerns were based upon the problems they had experienced with determining elemental concentrations and calibration of their instrument. Static-SIMS uses a primary ionic beam current that is about three orders of magnitude smaller than dynamic-SIMS. Therefore, static-SIMS analyzes only the upper atomic layers of the sample surface while dynamic hits the sample surface with more energy and is able to sputter off more sample material. Also, only about 2% of the sputtered material in static-SIMS is in the form of ions, which is what the mass spectrometer detects. Therefore, the amount of detectable material produced by the primary ion beam in static-SIMS is very small compared to dynamic-SIMS. The sensitivity of static SIMS varies among elements in the same matrix and for the same element in different matrices. This makes it difficult to use static-SIMS as a quantitative technique. It was upon these differences that the U.C. Berkeley/Lawrence Livermore group based their concerns. Because of the differences in the two instruments, comparison of samples analyzed on both machines would not have achieved our original intention of comparing two similar instruments. We also felt that our above comparison of ToF SIMS to a WD-EM was a more useful comparison that provided clear evidence that ToF SIMS can provide reasonable quantitative estimates of elemental composition, the true intent of instrument comparison. To our knowledge, we are the only ones using a ToF-SIMS for the analysis of otolith microchemistry.

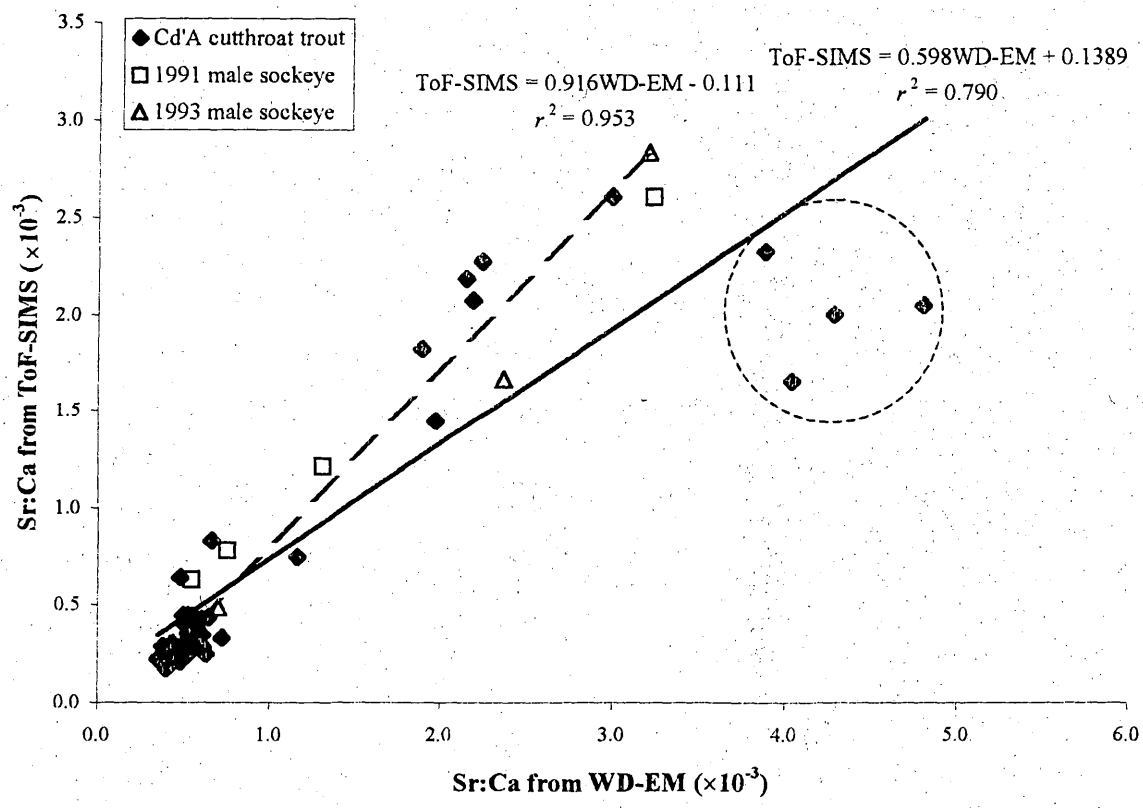

Figure 2 Comparison of ToF-SIMS Sr:Ca ratios to WD-EM Sr:Ca ratios. Samples include 19 otoliths from Coeur d'Alene River tributaries and two anadromous sockeye salmon otoliths (45 otolith-zone combinations). The solid line is the regression using all of the data. The dashed line is the regression of all of the data except for the edge zones of four Lavin Creek cutthroat trout (dashed circle).

2. Conduct analysis of salmonid otoliths from watersheds with different ranges in the Sr:Ca ratios of their tributaries to test efficacy of SIMS

We tested the ability of microchemistry to discriminate among trout occupying different tributaries using two complementary approaches. In the first approach, we measured Sr:Ca ratios of water samples from tributaries of two different watersheds, Coeur d'Alene River and Missouri River. Water sample data obtained from July 1997 in Phase One of our study indicated that the Sr:Ca ratios varied widely, ranging from 4.97×10^{-3} to 54.69×10^{-3} in the Coeur d'Alene tributaries. Though less variable, the Sr:Ca in water samples from the upper Missouri River and its tributaries, obtained in October 1997, also showed order of magnitude differences (2.40×10^{-3} to 8.30×10^{-3}). Water sample results were encouraging as they suggested that potential Sr:Ca ratio differences in freshwater were on the order of the large differences between fresh and saltwater, and thus pointed to the possibility of that microchemical differences in fresh waters would transfer to the otolith.

We conducted additional water sample analysis in 1999 with the primary objective of determining if there is a temporal and spatial variation in the Sr:Ca ratio of the water that could interfere with the detection of differences in the otolith microchemistry of fish from different streams. We also wished to test if water sampling could be a useful first step in determining if elemental concentrations in water were sufficiently different to translate into differences in otolith microchemistry. In short, where are differences in water chemistry different enough for there to be otolith microchemical differences, and where are they not?

A. Water analysis:

We collected three sets of water samples (20 sites per set) in the upper Missouri River system between Holter Dam and the town of Hardy, Cascade County, MT in 1999. These were selected based on the results from the 48 sites sampled in 1997, which were initially chosen by examining surface geology maps of the region. The tributaries sampled included: Little Prickly Pear Creek and two of its tributaries (Lyons Creek and Wolf Creek), the Dearborn River and Sheep Creek. To assess spatial variation in water chemistry, we obtained water samples from several sites within each tributary: near the mouth, in sections of the same tributary that had different geology, and below the confluence of secondary tributaries. On the mainstem Missouri River, water samples were collected at several sites between the uppermost tributary (Little Prickly Pear Creek) and below the lowermost tributary (Sheep Creek). To assess potential temporal differences in water chemistry in relation to flow, three replicate samples were collected from the same sites throughout the year. Water samples were collected in March, before any major snow melt and the streams were low, in May during spring runoff, and in October to replicate the original samples collected in the fall of 1997. A fourth sample was to be collected in 1999, during summer low flows, but this was not done because of higher flows due to rainstorms at that time.

Water samples were analyzed for Ca, Na, K and Mg at the Water Analysis Lab at the Rocky Mountain Research Station, Boise using an atomic absorption spectrophotometer (AAS). Because of the low levels of Sr in the water samples, they were also sent to the Soils Testing Lab at Montana State University, Bozeman for analysis using an inductively coupled plasma mass spectrometer.

There were no significant differences in the Sr:Ca ratios between pairs of samples collected at the same site at the same time for the March ($n = 15$, $P = 0.24$), May ($n = 15$, $P = 0.57$), and October ($n = 15$, $P = 0.40$) samples using a matched pair t-test. There were some interesting spatial differences in the 1999 water samples, however (Figure 3). The Sr:Ca ratios for both Little Prickly Pear Creek and the Dearborn River increased downstream. In addition, the tributaries appear to fall into two groups. The first consisting of Little Prickly Pear Creek, the Dearborn River, and Wolf Creek, and the second group consisting of Lyons Creek, Sheep Creek, and the Missouri River. An analysis of variance (ANOVA) and Bonferroni's multiple comparison procedure, using the Sr:Ca ratios as the response variable, indicated that the ratios were significantly different ($P < 0.05$) for most of the streams. However, Sr:Ca ratios were not significantly different between the Missouri River and both Lyons and Sheep Creeks ($P > 0.05$). The analysis also indicated that the ratios from Little Prickly Pear Creek and the Dearborn River were not significantly different ($P > 0.05$).

Temporal variation in Sr:Ca ratios of water samples was also detected. The Sr:Ca ratios from the March samples were significantly higher than from spring and fall samples (Bonferroni's multiple comparison procedure, $d.f. = 28$). The mean deviation of replicate samples collected in different seasons from the mean Sr:Ca ratios for that site was 7% but were as large as 20% in the Dearborn site. For the most part, the values among seasons, for a site were close together even though there were significant differences. This was particularly noticeable in Lyons Creek and was possibly a result of reduced surface runoff during winter. The Sr/Ca ratios from 1997 were on average twice the Sr/Ca ratios from 1999. In addition, the Sr/Ca ratios varied more within a tributary in 1997 than in 1999. These differences are most likely because AAS was used to analyze all elements in 1997, but in 1999 inductively coupled plasma emission spectrometry was used to analyze Sr. Strontium analyses were done on different instruments because it was found that AAS was not sensitive enough to accurately measure the low levels of strontium in our water samples. Therefore, a more sensitive instrument (ICP) was used.

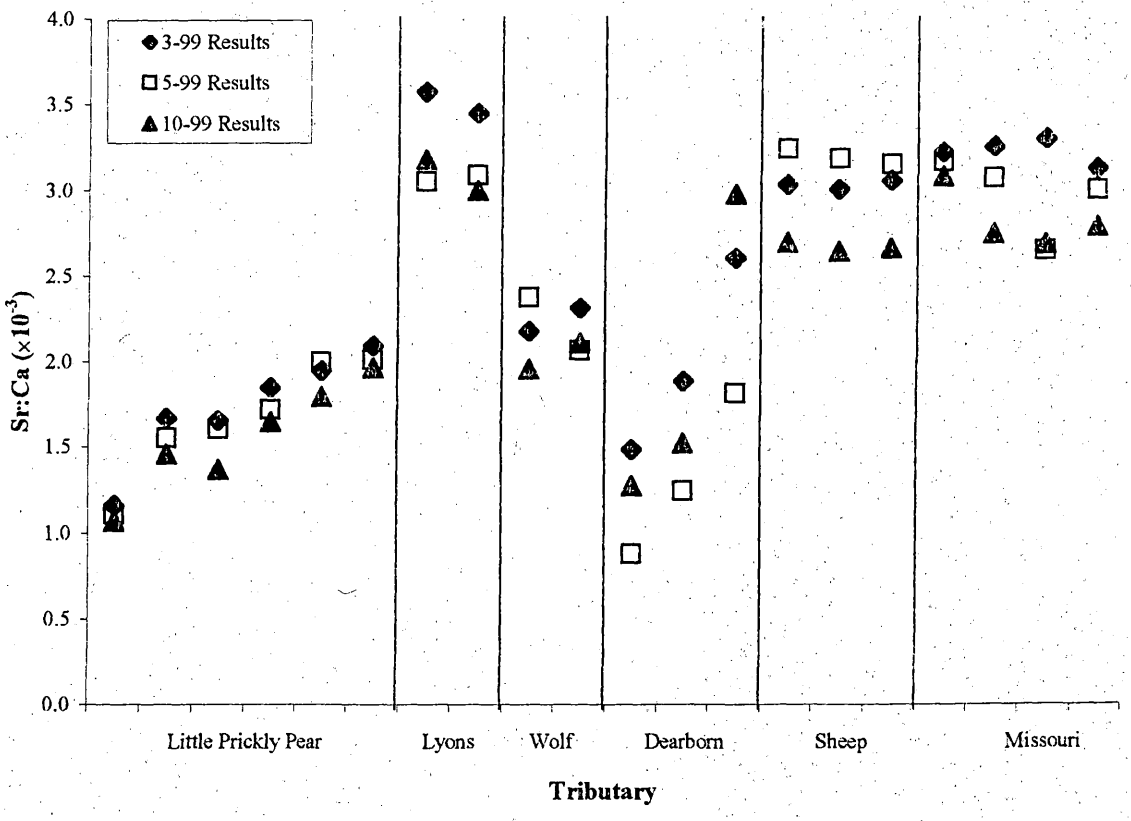

Figure 3 Sr:Ca ratios from the 1999 water samples collected from the upper Missouri River and its tributaries. The diamonds are the March samples, squares are the May samples, and triangles are the October samples. For each tributary, the sample sites on the right are further downstream.

B. Otolith Analysis:

Our second approach was to measure Sr:Ca ratios of otoliths of rainbow trout (Missouri River) and westslope cutthroat trout (Coeur d'Alene River) in these same tributaries. Our goal was to determine how sensitive ToF SIMS was in detecting differences among otolith composition of trout occupying different tributaries having varying differences in water chemistry. Otoliths were analyzed using ToF-SIMS from five fish from each of the seven tributaries, except for Iron Creek where otoliths from only four fish were analyzed. Missouri River fish were juvenile rainbow trout (age 1) collected from three tributaries (Little Prickly Pear Creek, the Dearborn River and Sheep Creek) in May 1998 at the tributary mouth as they migrated to the mainstem Missouri River. Cutthroat trout were captured in four tributaries of the Coeur d'Alene River (Canyon, Hudlow, Iron, and Lavin Creek). The cutthroat trout were between 1 and 3 years old but it was it was assumed that they had been spawned in the tributary in which they were captured.

Two zones were analyzed in each otolith. The Missouri River rainbow trout were analyzed in the area just outside of the nucleus and near the edge of the otolith. The site near the nucleus represents the growth shortly after hatching and the edge site represents growth around the time of capture. The Coeur d'Alene otoliths were also analyzed at the edge of each otolith. However, instead of analyzing the early growth zone of the fish, the nucleus was analyzed. The chemical composition of this zone is influenced by the environment that the female parent was in at the time of egg formation. The nucleus was analyzed, instead of the early growth, because the other otolith of the pair for each of these fish were analyzed in this zone using the WD-EM for Objective 1, part B of this study. As in part B, variation was tested by replicating assays both within zones and within an analysis site for a subsample of these otoliths.

Coeur d'Alene and Missouri River data were analyzed separately, but both were analyzed using similar models. The data were analyzed as a two-way factorial with the two factors being stream and otolith zone analyzed (e.g., early growth or edge for Missouri River rainbow trout). In addition to the two fixed factors, three random factors (fish, zone×fish interaction, and zone replication) were included in the model to account for variation from fish to fish, variation within a given zone of an otolith, and instrument variation. An α level of 0.05 was used for all significance tests.

The mean Sr:Ca ratio of cutthroat trout otoliths differed significantly among the four Coeur d'Alene tributaries ($F_{3,15} = 76.51$, $P = 0.00$; Figure 4). There was no significant zone ($F_{1,15} = 0.35$, $P = 0.56$) or interaction effect ($F_{3,15} = 0.85$, $P = 0.49$). Lavin Creek cutthroat trout had significantly higher Sr:Ca ratios than other streams for both nucleus and edge zones (Bonferroni's multiple comparison procedure $P < 0.01$, $d.f. = 15$). In contrast, there was no significant difference among Sr:Ca ratios for the three other streams (Canyon, Hudlow, and Iron creeks).

The mean Sr:Ca ratios of rainbow trout were significantly differently among the three Missouri River tributaries ($F_{2,12} = 4.14$, $P = 0.04$; Figure 5). While the mean Sr:Ca ratio of Sheep Creek rainbow trout otoliths was higher than the other two tributaries, there were no

significant differences found with pair-wise tests using Bonferroni's multiple comparison procedure. This contradiction in the results of the F-test and the multiple comparison tests is because the F-test calculates a pooled standard error, while the multiple comparison tests use the standard error calculated for each group separately. An increase in the number of samples would probably resolve this conflict (see Objective 3). There was also a significant zone effect ($F_{1,12} = 6.75$, $P = 0.02$). Again, the multiple comparison tests did not show any significant differences despite the mean Sr:Ca in the early growth zones being generally higher than the Sr:Ca of the edge zone for each of the tributaries. There was no significant interaction effect.

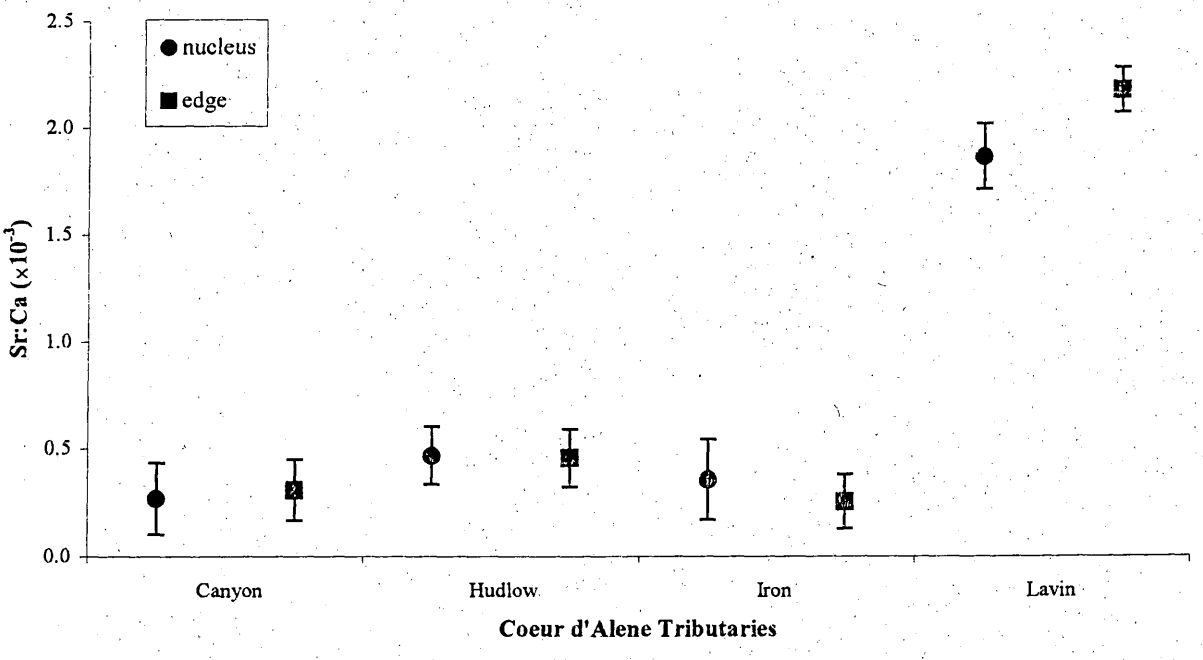

Figure 4 Mean otolith Sr:Ca ($\times 10^{-3}$) for cutthroat trout from the Coeur d'Alene River tributaries. Circles are the nucleus Sr:Ca ratios and squares are the edge Sr:Ca ratios. Error bars are ± 1 standard error.

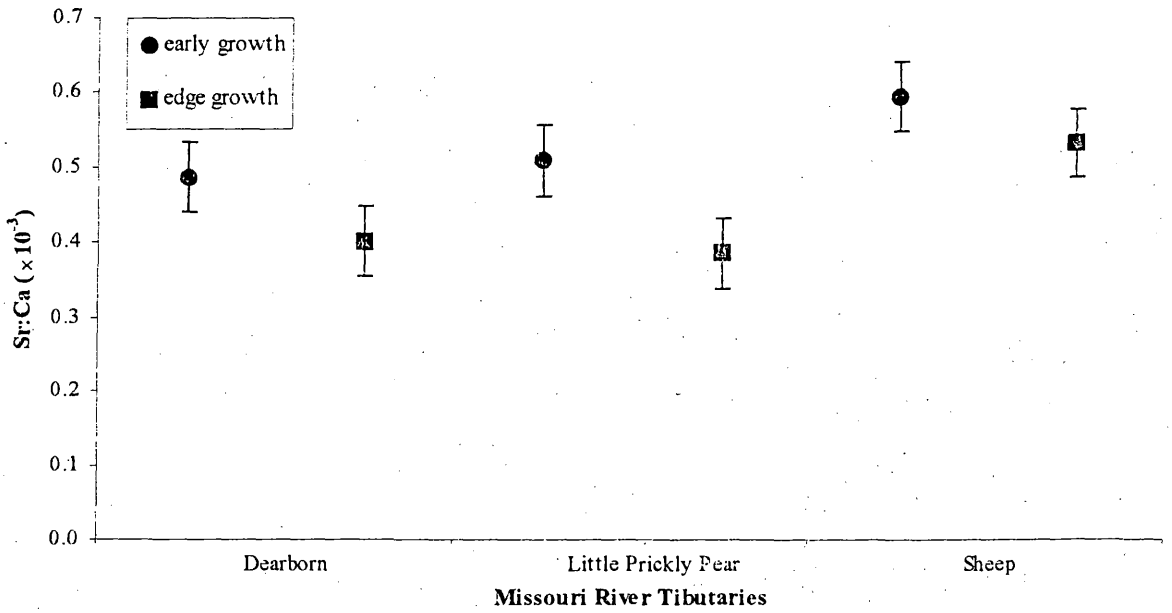

Figure 5 Mean otolith Sr:Ca ($\times 10^{-3}$) for rainbow trout collected in 1998 from the Missouri River tributaries. Circles are the early growth Sr:Ca ratios and squares are the edge Sr:Ca ratios. Error bars are ± 1 standard error.

3. Test variation of Sr:Ca ratio in otoliths over time within a single system

An additional factor with regards to the efficacy of ToF-SIMS to identify life history in inland salmonids revolves around the question of how microchemistry on otoliths may vary over time. Recent studies of the microchemical composition of otoliths to separate fish stocks suggest that the level of strontium remains relatively constant from year to year, unlike other elements such as Zn, Fe and Mn. However, our water chemistry results suggest that seasonal changes in Sr:Ca may occur within the same tributary. The purpose of this objective was to determine the annual and seasonal variation in otolith Sr:Ca ratios. Because we did not find significant differences among all spawning tributaries and the majority of juvenile rainbow trout migrate to the mainstem Missouri River in late spring/early summer, we focused our efforts on testing for annual variation in otolith Sr:Ca ratios

To test for yearly variation in the otolith Sr:Ca ratio of fish from the Missouri River tributaries, Sr:Ca ratios from rainbow trout otoliths were compared between 1998 and 1999. As in 1998, otoliths were of age-1 rainbow trout were obtained from 15 rainbow trout, 5 each from Little Prickly Pear Creek, the Dearborn River and Sheep Creek. These otoliths were analyzed using the ToF-SIMS and the same procedures as described in Objective 2. The data from this set of otoliths were added to the data from the 15 Missouri Rainbow trout in Objective 2 and were analyzed using the same ANOVA model as in Objective 2, but with the

year the fish were collected was added as a fixed effect. Therefore, a total of 10 otoliths from each tributary (5 from May 1998 and 5 from May 1999) were used for statistical analysis.

We found no significant differences in Sr:Ca between years ($F_{1,25} = 2.04$, $P = 0.17$). In addition, we also found that the mean otolith Sr:Ca ratio differed significantly among the tributaries ($F_{2,24} = 26.51$, $P = 0.00$; Figure 6), with the mean Sr:Ca for Sheep Creek fish significantly higher than the other two tributaries for both years, whether years were combined or separate. The was also a significant zone effect ($F_{1,24} = 122.76$, $P = 0.00$) and a stream-year interaction effect ($F_{2,25} = 2.04$, $P = 0.03$). As

Because of the inability to differentiate among all three Missouri River tributaries using only Sr:Ca ratios, we are also exploring whether other elements would be more discriminatory. As noted, one of the distinct advantages of ToF-SIMS over other microchemical methods is the ability to collect multiple elemental spectra simultaneously. Thus far we have examined elemental counts for Fe, Li, Mg, and Zn, but none have shown any promising results as yet.

4. Test identification of tributary of origin in adult salmonids using otolith microchemistry

Beginning in spring of 1998, yearling and young of the year rainbow trout migrating from the Missouri River tributaries were captured and batched tagged with coded wire tags. The tags were placed in different body locations to indicate the tributary from which they were migrating. The year that the fish was tagged was identified by using a different type of coded wire tag for each year (i.e. blank tags – 1998; binary tags – 1999; decimal tags – 2000). Some of these fish were recaptured in the mainstem Missouri during the annual fall population estimate. The intention of this objective was to analyze tagged fish of known origin and non-tagged fish to determine the timing of entry into the mainstem Missouri River and to determine the tributary of origin of individual fish using Sr:Ca ratios. Because we did not find significant differences among all spawning tributaries with known-origin juveniles, we did not continue our testing with unknown-origin adults. However, we did measure Sr:Ca ratio in tagged fish recaptured in the mainstem Missouri to assess how well the ratio could correctly predict tributary of origin (Sheep Creek or Little Prickly Pear/Dearborn River).

Between 1998 and 2000, 123 tagged rainbow trout from Little Prickly Pear Creek, the Dearborn River and Sheep Creek were recaptured during the annual fall population estimates. The otoliths of 9 of these fish (3 from each tributary) were analyzed using ToF-SIMS. All of these fish had been tagged in 1998 and were recaptured 5 to 29 months after being tagged. Six of these fish migrated, and were tagged, as yearlings. The other three fish migrated as young of the year. Microchemical assays were done in both the early growth zone and the edge of the otolith in all fish. Transects were also done for two of the fish; one of the fish migrated from the Dearborn River and the other from Little Prickly Pear Creek.

The Sr:Ca ratios of the early growth zone were significantly different among streams (ANOVA, $F_{1,8} = 59.77$, $P = 0.00$; Figure 8). Two of the three fish that migrated from Sheep Creek had significantly higher early growth Sr:Ca ratios than fish that had migrated from either Little Prickly Pear Creek or the Dearborn River (Bonferroni's multiple comparison

procedure, $d.f = 8$). There were no significant differences in the early growth Sr:Ca of fish that had migrated from either Little Prickly Pear, or the Dearborn River, except for LP2. Visual comparison of the mean Sr:Ca ratio of the fish analyzed in this objective to the mean otolith Sr:Ca ratio for each of the three tributaries, determined in Objective 3, show a similar pattern in that in both sets of data, Sheep Creek fish had the highest Sr:Ca ratios and Dearborn River fish with the lowest (Figure 7). However, the mean Sr:Ca ratios estimated in Objective 3 are lower than the early growth Sr:Ca of fish that migrated from Sheep Creek. The same was true for Little Prickly Pear Sr:Ca ratios.

The two otoliths in which transect analyses were made showed changes in the Sr:Ca ratios along the growth axis indicative of migration into the mainstem Missouri River (Figure 8). Both fish were tagged in 1998 at age 1 as they migrated from their respective tributaries in late spring/early summer). These fish were later recaptured in the Missouri River. The fish that migrated from the Dearborn River was recaptured in October 2000, over 2 yrs after being tagged. The transect shows an increase in Sr:Ca from a mean of 0.37×10^{-3} (SD = 0.07) to 0.62×10^{-3} (SD = 0.01) shortly after the first annulus was formed. The other fish migrated from Little Prickly Pear Creek and was recaptured in October 1998, about 5 months after being tagged. As in the other rainbow trout, the Sr:Ca ratio increased from 0.48×10^{-3} (SD = 0.05) to 0.72×10^{-3} (SD = 0.01) after the age-1 annulus. We could be certain that the change in the Sr:Ca ratios were a result of migration to the mainstem Missouri River because the water samples from the Missouri River had significantly higher Sr:Ca ratios than both Little Prickly Pear Creek and the Dearborn River (Figure 3).

An interesting pattern was seen in the first few daily rings just outside of the nucleus second fish. The Sr:Ca ratio of this site was higher than the Sr:Ca ratio of the other sites that were located before the first annulus. The Sr:Ca ratio then decreases within 60 μm. Because this assay was located within the first week or two of hatching, the Sr:Ca ratio at this site probably was not influenced by the environment in which female parent was during egg formation. One explanation for the change in the Sr:Ca ratio, is that this fish was spawned in Lyons Creek, a tributary to Little Prickly Pear Creek, and migrated to Little Prickly Pear shortly after hatching. This is a possible scenario because the 1999 water samples showed that Lyons Creek had a significantly higher Sr:Ca than Little Prickly Pear Creek (Figure 3).

The analysis of the adult rainbow trout of known tributary origin showed that, like the juvenile fish analyzed in Objective 2 and 3, the fish of Sheep Creek origin had higher otolith Sr:Ca ratios than fish of Little Prickly Pear or Dearborn River origin even after having migrated from their natal stream and spending several months to years in the mainstem Missouri River. In some cases, the mean Sr:Ca ratio of the adult fish was similar to the mean Sr:Ca ratio of juvenile fish of known origin from the same year class. The transects also showed that ToF-SIMS can be used to track environmental changes that a fish experiences and that these changes can be combined with the age and growth information also contained in their otoliths to give a more complete picture of an individual fish's life history.

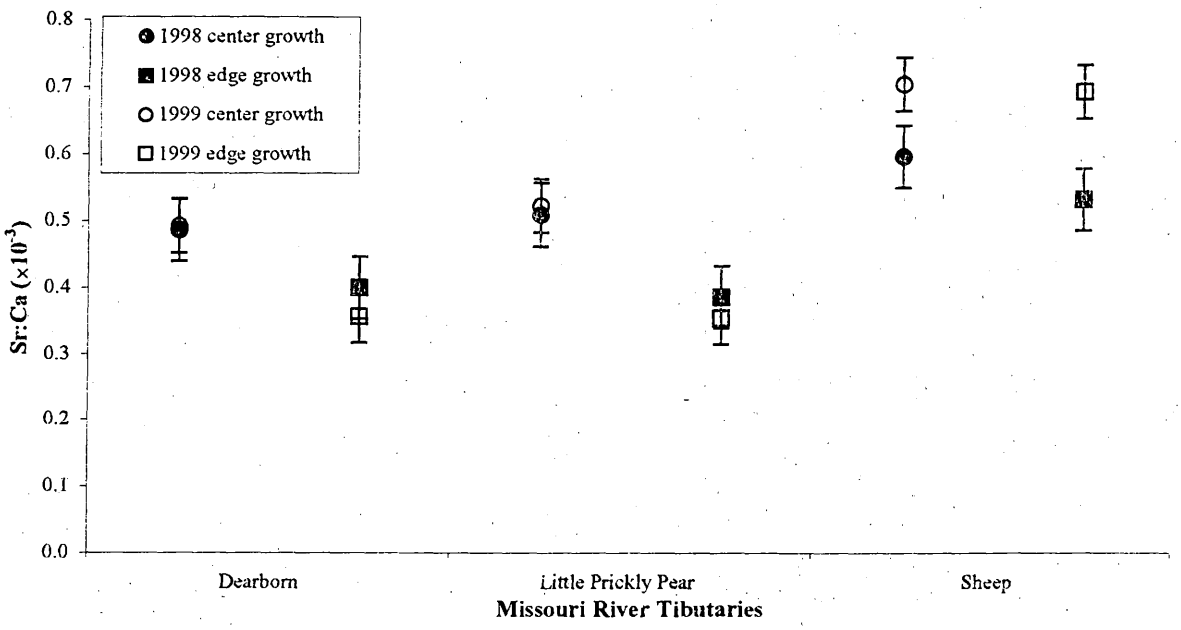

Figure 6 Mean otolith Sr:Ca ($\times 10^{-3}$) for rainbow trout collected in 1998 and 1999 from the Missouri River tributaries. Circles are the Sr:Ca for the early growth assays and squares are the Sr:Ca for the edge growth zone. Error bars are ± 1 standard error.

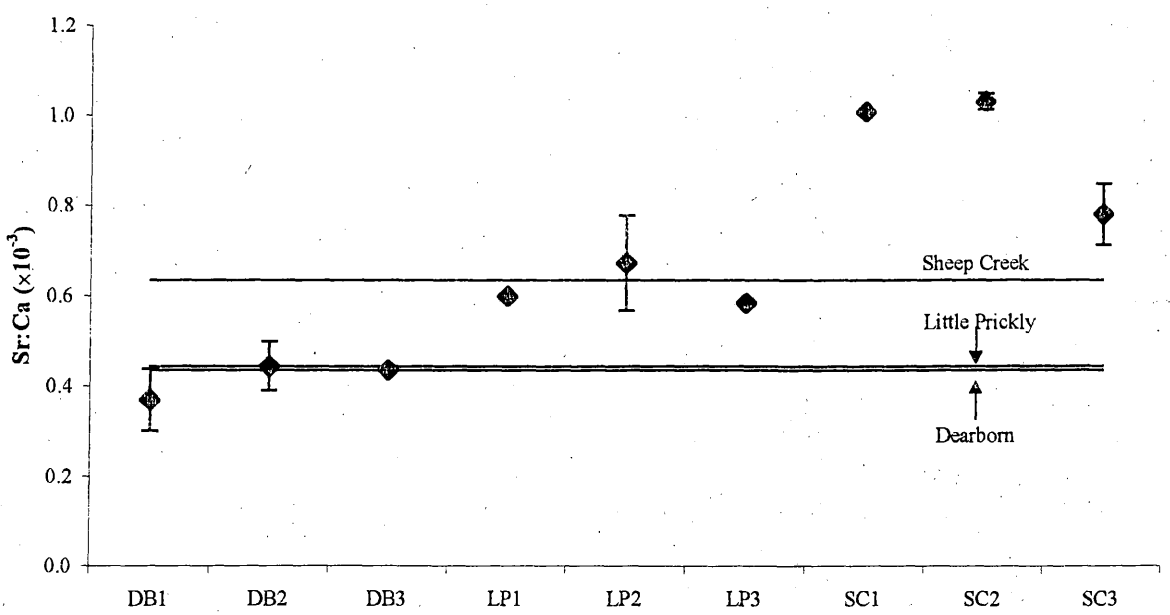

Figure 7 Mean early growth Sr:Ca ratios for Missouri River rainbow trout that were tagged as juveniles migrating from the spawning tributaries and later recaptured. The x axis labels designate the tributary of origin; DB = Dearborn River, LP = Little Prickly Pear, SC = Sheep. The solid lines are the mean otolith Sr:Ca ratios of each of the tributaries as determined in Objective 3. Error bars are ± 1 standard error.

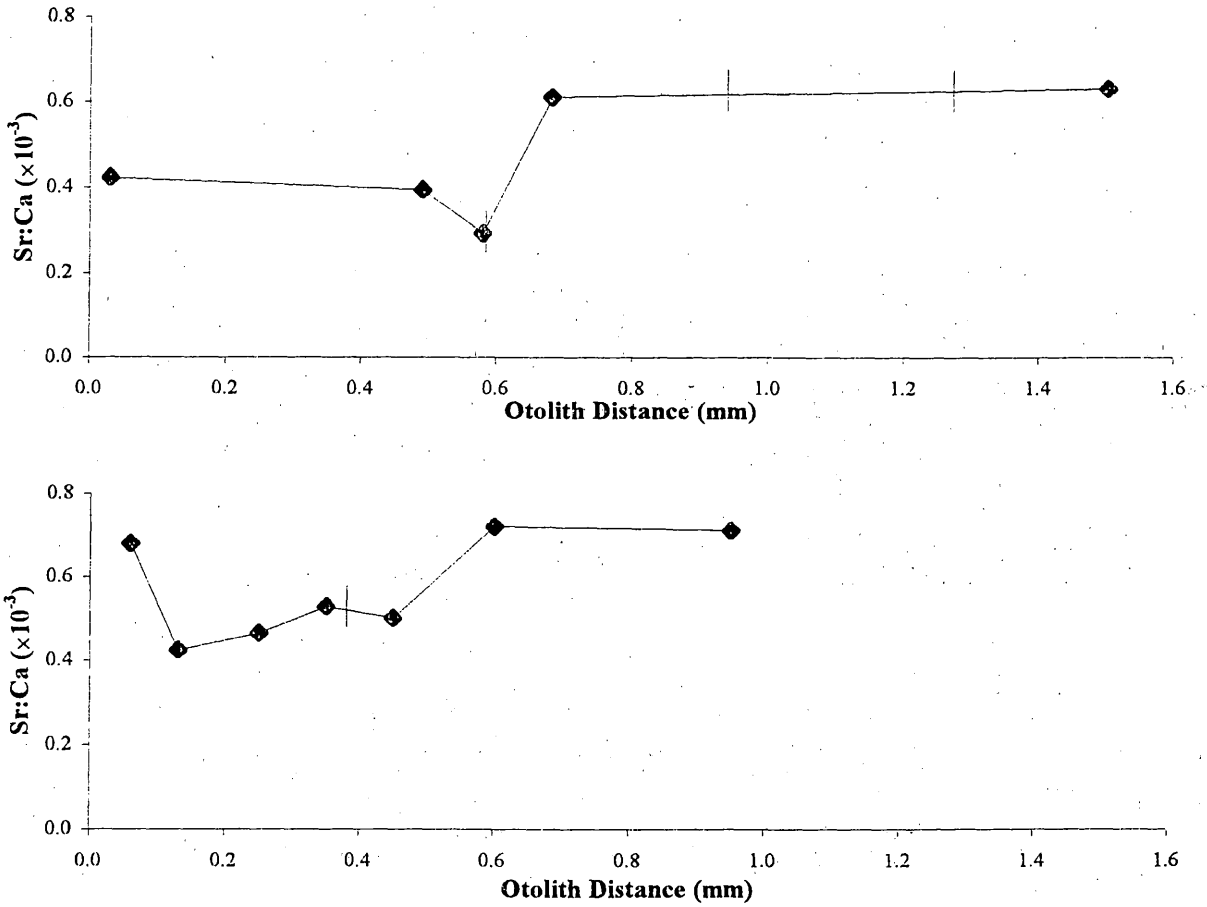

Figure 8 Transect plots of Sr:Ca ratios for two Missouri River rainbow trout that were tagged as yearlings migrating from the tributaries in 1998 and later recaptured in the mainstem. Top transect is a 3 year old fish from the Dearborn River recaptured in 2000. Bottom transect is a 1 year old fish from Little Prickly Pear Creek recaptured in the fall of 1998 in the Missouri River. Vertical bars indicate location of annuli.

5. Investigate whether strontium isotope ratios can be used to determine life history patterns of interior salmonids

Stable isotope ratios have long been established as being useful geologic and biologic tracers, recorders of the environment (e.g. temperature) and indicators of feeding history, trophic level, and metabolic rate (Campana 1999). In fish otoliths, stable isotope ratios have been used to reconstruct temperature histories, differentiate among groups of fish, and reconstruct migration patterns (see Campana 1999 for examples). Most of the past stable isotope work in otolith microchemistry has focused on O and C; however, Sr isotopes, among other elements, are increasingly being used. For example, Kennedy et al. (1997, 1999) and Ingram and Weber (1999) used Sr isotopes as markers to identify the origin of salmonids. The isotopes ^{87}Sr and ^{86}Sr are used to construct the Sr isotope ratios because ^{87}Sr is a product of the

radioactive decay of ^{87}Rb and the amount of ^{86}Sr does not change over time; therefore, older rocks and (or) rocks with higher Rb:Sr ratios have higher ^{87}Sr:^{86}Sr ratios (Kennedy et al. 1999). The advantage of using Sr isotope ratios is that they are incorporated into the hard parts of the fish in the same ratio as the water and, unlike elemental concentrations, isotope ratios are not influenced by physical and biological factors (Kennedy et al 1999). Therefore, Sr isotope ratios offer another possibility for determining life history patterns of interior salmonids.

We investigated the potential use of Sr isotope ratios for this study. Water samples from the upper Missouri River and its tributaries were collected at a subset of the water sampling sites in Objective 2, in May and October 1999. We had planned on analyzing samples for Sr isotope concentrations, but results from our ToF-SIMS analysis showed that this instrument is not accurate enough for detailed discrimination of different isotopes. This is because the naturally occurring Sr isotopes, ^{87}Sr and ^{86}Sr are low in abundance (7.00 and 9.86 %, respectively) compared to the 82.58 % occurrence of ^{88}Sr and a very accurate quantification of the isotopic concentrations are needed. While ToF-SIMS is able to detect the presence of the isotopes, the quantitative abilities of ToF-SIMS for the needed power of discrimination have not yet been fully developed. We were able to use the ratios of Ca as an internal check of the instrument tuning because of the predominance of Ca in otoliths. Kennedy et al. (1997, 1999) and Ingram and Weber (1999) both used thermal ionization mass spectrometry, a bulk solution method, in their Sr isotopic studies. An accuracy of five to six decimal places is required to properly do these types of analyses (D. Mogk, Montana State University, MT, personal communication), and as yet we know of no microprobe method to accurately measure Sr isotopes.

As a confirmation of the lack of ability of ToF-SIMS to effectively be used to measure the Sr isotope in fish otoliths, we measured the ^{87}Sr and ^{86}Sr ion counts in the otoliths of the 15 juvenile rainbow trout from Little Prickly Pear Creek, the Dearborn River, and Sheep Creek, collected in May 1999 (54 assays). Measurement of the isotope peaks in the spectra was difficult because of possible overlapping caused by other elements or compounds that have similar mass as Sr isotopes that we were measuring, such as ^{87}Rb. The mean ^{87}Sr:^{86}Sr for the assays of these otoliths was 0.6899 (SD = 0.2510); the ^{87}Sr:^{86}Sr ratio, based on natural abundances, is 0.1194. Analysis of the ^{87}Sr:^{86}Sr for these fish, using the same model as in Objective 3 with the isotopic ratio as the response variable, indicated that there was no significant difference in the ^{87}Sr:^{86}Sr amongst the streams ($F_{2,12} = 0.97$, P = 0.4053). Also, the average ^{87}Sr:^{86}Sr for these fish was 0.9791 (SD = 0.1011). This is much higher than the value of the Sr isotope ratios reported in either Kennedy (1999) or Ingram and Weber (1999) for both freshwater and otoliths. The highest ratio reported was 0.731 in Kennedy et al (1999).

Summary

Otolith microchemistry is a relatively new field in fisheries science and is increasingly being applied to freshwater fisheries management. One of the problems with freshwater application of these methods has been the lack of instruments sensitive enough to detect the small differences in elemental concentrations often found in freshwater systems. To our knowledge, other than this study ToF-SIMS had not been used for microchemical analysis of otoliths. However, secondary ion mass spectrometry was identified as a technique that is more sensitive than electron microprobes (Campana 1997). The goal of this study was to evaluate the efficacy of ToF-SIMS for use in otolith microchemistry. To fully evaluate ToF-SIMS objectives included: 1) testing the precision and accuracy of ToF-SIMS using artificial "standard" otoliths and comparing microprobe methods; 2) analyze otoliths from watersheds with different ranges in the tributary Sr:Ca ratios; 3) test variation of Sr:Ca ratio over time; 4) test the ability to identify tributary of origin in adult salmonids; and 5) investigate the use of Sr isotopes to determine life history patterns.

Because ToF-SIMS has not been used previously to analyze otoliths, much time was spent on the development of sample preparation and analysis methods. ToF-SIMS is a relatively new instrument and its use in otolith microchemistry represented a new application. Because of this, much needed to be learned by both the investigators in this study and the members of ICAL. Progress was occasionally hampered by instrument problems and lack of complete understanding of the instrument. However, after many setbacks we now have a much more complete understanding of all that is involved in analyzing otoliths using ToF-SIMS. Development of the artificial "standard" otoliths was very useful in helping to determine if the instrument was properly tuned. The sometimes high variation we observed between sampling sessions suggests that use of a standard at the start of each session should be incorporated in all otolith microchemistry studies as a means of instrument validation.

In this study we were able to demonstrate that ToF-SIMS is a comparable instrument to WD-EM. Although we were not able to analyze samples of known Sr:Ca ratios, we did compare ToF-SIMS results to the WD-EM, an instrument that does have quantitative abilities. Therefore, the comparison of the Coeur d'Alene cutthroat trout otoliths acted as a surrogate method of testing the accuracy of ToF-SIMS. We found that there was a nearly 1:1 relation between the Sr:Ca ratios obtained using ToF-SIMS and WD-EM. This suggests that there is little bias in the ToF-SIMS analyses, compared to WD-EM. We also found that on average the ToF-SIMS Sr:Ca ratios were less variable than WD-EM Sr:Ca ratios in the cutthroat trout analyses both within sites and among sites within a given growth zone. This suggests ToF-SIMS may have greater discriminatory ability than WD-EM which may be particularly useful in fresh waters where Sr:Ca differences may be slight.

Water chemistry analyses indicated that there were both spatial and temporal differences in Sr:Ca ratios among some of the tributaries of interest. These differences could interfere with the discrimination of fish from different tributaries. Otolith Sr:Ca ratios typically showed a similar pattern as the water chemistry data (i.e. the fish from streams with high Sr:Ca ratios typically had high otolith Sr:Ca ratios). There is a strong correlation between otolith Sr:Ca ratios from fish from the Missouri River tributaries and water Sr:Ca ratios from the same

tributaries, collected within the same month (Figure 9). The slope of this relation in the regression (0.15) was nearly identical to the 0.14 slope that Campana (1999) reported for the relation between ambient water and otolith Sr:Ca, which was based on the data reported in five published studies. Three of the studies used marine species, including golden perch *Macquaria ambigu*, trout cod *Maccullochella macariensis*, *Girella elevata*, and temperate damselfish *Parma microlepis*. The other two studies were conducted using chum salmon *O. keta*, sockeye salmon and juvenile tilapia *Oreochromis niloticus*. This positive relation indicates that the otolith Sr:Ca is positively related to the Sr:Ca of the environment that the fish is living in and that this difference is able to be detected by ToF-SIMS. Therefore, initial water samples taken before otolith analysis, can be very useful in providing a reliable index of otolith Sr:Ca ratios.

The ability of ToF-SIMS to differentiate fish from different tributaries was somewhat limited due to only slight differences in Sr:Ca in the water. In both the Coeur d'Alene River cutthroat trout and the Missouri River rainbow trout there was one tributary that showed significant differences in otolith Sr:Ca. However, it was difficult to consistently differentiate these fish from the other fish because of variation in Sr:Ca ratios both among fish from the same tributary. Estimated variances between fish were smaller for the Missouri River rainbow trout than either the variance between the two zones analyzed in the juvenile fish or within the zones (Table 2). This means that there appears to be more variation within individual fish than among different fish from the same tributary. For the Missouri River fish, we were able to differentiate Sheep Creek fish (mean Sr:Ca = 0.63×10^{-3}) from Little Prickly Pear fish (mean Sr:Ca = 0.44×10^{-3}) and Dearborn River fish (mean Sr:Ca = 0.43×10^{-3}) using the combined 1998 and 1999 samples from each tributary. The difference between the mean Sr:Ca ratio for Sheep Creek fish and Little Prickly Pear fish was 0.19×10^{-3}. This was the smallest difference in mean otolith Sr:Ca ratios in which we found statistical differences. With this information and the relation between otolith Sr:Ca and water Sr:Ca, we can then estimate the approximate difference that would be needed in the Sr:Ca ratio of the water in the tributaries of interest to translate to a detectable difference in otolith Sr:Ca among streams. This is done by dividing the minimum detectable difference in otolith Sr:Ca by the slope of the otolith-water Sr:Ca ratio relation. Assuming, the difference between the mean Sheep Creek and Little Prickly Pear otolith Sr:Ca ratios is the is the minimum needed to differentiate fish among tributaries, using ToF-SIMS, we can estimate the approximate difference in the water Sr:Ca ratios would need to be 0.34×10^{-3} (0.19×10^{-3} /0.1534).

This study has demonstrated that ToF-SIMS has the sensitivity and repeatability for detailed analysis of otolith microchemical composition in freshwater systems. However, some aspects need to be studied further; for example, the quantitative abilities of ToF-SIMS. Currently, little is known about how the elements of interest behave in otoliths when present in different concentrations. In other words, does the rate of ejection from the sample surface change as the concentration of the element changes? The hurdles that we have encountered are not unusual when trying to develop new analysis techniques for an instrument that was primarily designed for other applications. In the future ToF-SIMS may be used regularly in the field of otolith microchemistry. One of the major disadvantages of ToF-SIMS is the cost of analysis ($45-75/hr). This is a factor because it takes 9 minutes to collect a single spectrum (4 min sputter and 5 min data acquisition). In addition, a technician needs to be

present during the analysis session. One cannot pre-program the analysis locations and let the instrument run unattended like WD-EM. Part of the current high cost of ToF-SIMS is the fact that it is a relatively new instrument, there are very few of them in existence, and they are in high demand. However, the advantages of ToF-SIMS over some of the other microprobe instruments currently being are still its sensitivity, ability to collect information on most elements with a single assay, and spatial capabilities (0.5-1.0 μm beam diameter).

Table 2 Table of estimated variances in Sr:Ca ratios among fish, between the zones analyzed and within zones. Data are from the 1998 and 1999 Missouri River juvenile rainbow trout.

Variance	1998	1999
Among fish	0.001300	0.001594
Between zones	0.006528	0.003627
Within zones	0.002624	0.002908

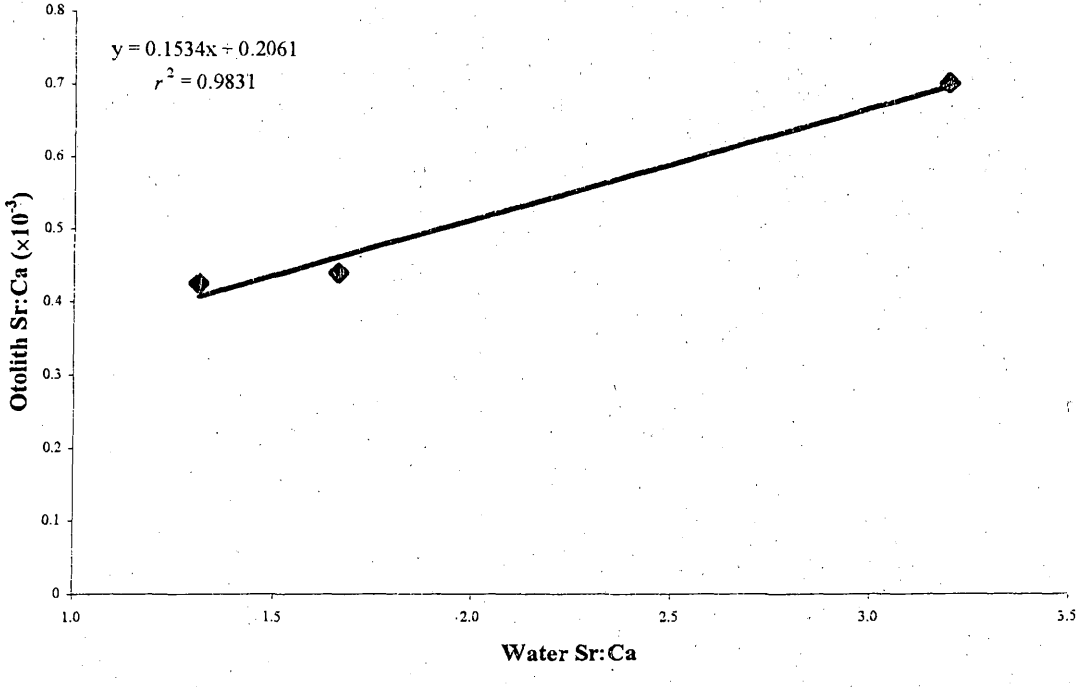

Figure 9 Relation of Sr:Ca ratios of Missouri River rainbow trout otoliths to water samples collected from the same tributaries. Both the fish and the water samples were collected in May, 1999.

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